Batch and continuous culture-based selection strategies for acetic acid tolerance in xylose-fermenting Saccharomyces cerevisiae

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Abstract: Acetic acid tolerance of Saccharomyces cerevisiae is crucial for the production of bioethanol and other bulk chemicals from lignocellulosic plant-biomass hydrolysates, especially at a low pH. This study explores two evolutionary engineering strategies for the improvement of acetic acid tolerance of the xylose-fermenting S. cerevisiae RWB218, whose anaerobic growth on xylose at pH 4 is inhibited at acetic acid concentrations >1 g L(-1) : (1) sequential anaerobic, batch cultivation (pH 4) at increasing acetic acid concentrations and (2) prolonged anaerobic continuous cultivation without pH control, in which acidification by ammonium assimilation generates selective pressure for acetic acid tolerance. After c. 400 generations, the sequential-batch and continuous selection cultures grew on xylose at pH 4 with 6 and 5 g L(-1) acetic acid, respectively. In the continuous cultures, the specific xylose-consumption rate had increased by 75% to 1.7 g xylose g(-1) biomass h(-1) . After storage of samples from both selection experiments at -80 °C and cultivation without acetic acid, they failed to grow on xylose at pH 4 in the presence of 5 g L(-1) acetic acid. Characterization in chemostat cultures with linear acetic acid gradients demonstrated an acetate-inducible acetic acid tolerance in samples from the continuous selection protocol.

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Batch and continuous culture-based selection strategies for acetic-acid tolerance in xylose-fermenting *Saccharomyces cerevisiae*

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Abstract

Acetic-acid tolerance of *Saccharomyces cerevisiae* is crucial for yeast-based production of bioethanol and other bulk chemicals from lignocellulosic plant-biomass hydrolysates. Acetic acid released during hydrolysis inhibits yeast growth and metabolism, especially at low pH. Targeted metabolic engineering is hindered by the complex, multi-factorial nature of acetic-acid tolerance. This study explores two evolutionary engineering strategies for improvement of acetic-acid tolerance of the xylose-fermenting strain *S. cerevisiae* RWB218, whose anaerobic growth on xylose at pH 4 is inhibited at acetic-acid concentrations above 1 g L^{-1}: (i) sequential anaerobic, pH-controlled batch cultivation (pH 4) at increasing acetic-acid concentrations, and (ii) prolonged cultivation in anaerobic continuous cultures without pH control, in which acidification caused by ammonium assimilation generates a selective pressure for improved acetic-acid tolerance. After ca. 400 generations, the sequential-batch and continuous selection cultures grew on xylose at pH $\leq$ 4 in the presence of 6 g L^{-1} and 5 g L^{-1} acetic acid, respectively. In the continuous cultures, the specific xylose-consumption rate had increased by 75% to 1.7 g xylose (g biomass)$^{-1}$ h^{-1}. After storage of samples from both selection experiments at -80 ºC and cultivation in the absence of acetic acid, they failed to grow on xylose at pH 4 in the presence of 5 g L^{-1} acetic acid. Characterization in chemostat cultures with linear acetic-acid feeding gradients demonstrated a strong acetate-inducible acetic-acid tolerance in samples from the continuous selection protocol. This evolved culture provides a valuable platform for analysis and improvement of acetic acid tolerance and its regulation.

Introduction

Evolutionary engineering is a rational approach for obtaining microorganisms with industrially desirable phenotypes (Sauer 2001), based on mutation and selection. Of the frequent mutations that occur within microbial cultures, some enable the host cell to grow and reproduce more effectively. The growth environment largely determines whether and to what extent a particular mutation and its resulting phenotype are beneficial to the host. The environment is thereby said to "select" certain phenotypes. The key challenge of the evolutionary engineer is to design, test and develop cultivation strategies that effectively select cells with desirable phenotypes. For selection to occur, at least two mutants with different phenotypes must be present, either from the onset of the culture, or arising during cultivation. Selection of desirable microbial phenotypes can be 'artificial' (i.e. using man-made devices such as colony pickers or cell sorters (Valli et al. 2006)), or 'natural', by allowing mixtures of cells with differing phenotypes to compete for common resources during cultivation. Herein, we use the term 'selection' to refer exclusively to the latter.

A brief description of two popular cultivation techniques illustrates how laboratory cultivation can be used to select for particular phenotypes. In a typical chemostat culture, a single growth-limiting nutrient is continuously present at a low concentration (Daran-Lapujade et al. 2008; Novick and Szilard 1950). All else being constant over time, long-term chemostat cultivation will therefore select for cells with an increased affinity, i.e. cells that can achieve a higher specific growth rate at a given suboptimal concentration of the growth-limiting nutrient. Conversely, in a typical batch culture, all nutrients are initially in excess, and nutrient limitation only occurs briefly before the culture enters stationary phase due to nutrient depletion. Consequently, selection in batch cultures will tend to favor cells that can grow fast at non-limiting substrate concentrations. Selection strategies may be further improved by, for example, the application of dynamic feeding regimes (Wisselink et al. 2009) or by the application of chemical or physical stresses (Almeida and Hahn-Hagerdal 2009).

A combination of evolutionary engineering in batch and chemostat cultures has been applied successfully to improve the kinetics of xylose- and arabinose-fermentation by genetically engineered strains of bakers' yeast (*Saccharomyces cerevisiae*) (Kuyper et al. 2005; Sonderegger and Sauer 2003; Wisselink et al. 2009), with the goal to enable fuel ethanol production from non-food lignocellulosic plant biomass. However, fermentation of these pentose sugars is not the only challenge for yeast-based ethanol production from such feedstocks. The reason is that several inhibitors of yeast growth and metabolism are released during hydrolysis of lignocellulose (Klinke et al. 2004; Palmqvist et al. 1999). A particularly important inhibitor in lignocellulosic hydrolysates is acetic acid, which is released upon hydrolysis of acetyl groups from the carbohydrate polymers present in plant biomass (Lima et al. 2004; Palmqvist and Hahn-Hägerdal 2000; Zaldivar et al. 2001). Especially at low pH, acetic acid is a strong inhibitor of microbial metabolism and growth, which explains its common use as a food preservative. Development of pentose-fermenting yeast strains with an improved tolerance to acetic acid offers an interesting approach to the seemingly unavoidable presence of acetic acid in lignocellulosic hydrolysates.

Acetic acid is a weak organic acid (pK = 4.76). Under conditions relevant for yeast cultivation, it exists in two forms – protonated and unprotonated. The protonated form is relatively non-polar, which allows it to passively diffuse across the (hydrophobic) plasma membrane. Alternatively, acetic acid can enter yeast cells via the Fps1p aquaglyceroporin (Mollapour and Piper 2007; Mollapour et al. 2008). Independent of the mechanism of entry into the cytosol, where the pH is near-neutral, dissociation into a proton and acetate ion occurs. Intracellular accumulation of protons and acetate anions can interfere with the function of some enzymes (Pampulha and Loureiro-Dias 1990), thus causing inhibition of metabolism and growth. Many microbes, including *S. cerevisiae*, have transmembrane proteins that expel protons and organic anions from the cytosol. These generally require a net input of free energy to drive ion export, e.g. via ATP hydrolysis (Pampulha and Loureiro-Dias 1990; Piper et al. 2001; Thomas et al. 2002). At a low extracellular pH, exported acetate and protons may reassociate and diffuse back into the cell, leading to a cyclic

process in which the plasma membrane proton-motive force is dissipated. Competition of (cyclic) energy-dependent ion transport with free-energy-requiring, growth-related cellular processes is likely to contribute to growth inhibition by acetic acid.

Based on current knowledge on acetic acid inhibition, several metabolic engineering approaches might be envisaged to improved tolerance to acetic acid. For example, characteristics of the plasma membrane could be altered to decrease the rate of diffusion of acetic acid into the cytosol, diffussion facilitating proteins can be deleted (Mollapour et al. 2008), cytosolic proteins might be altered to tolerate higher intracellular concentrations of protons and acetate ions, or the rate of ion export could be increased by altering the number, type, or activity of proton and acetate exporters in the membrane. Furthermore, ATP availability could be increased by increasing the sugar consumption flux, or by reducing the ATP requirement of other cellular processes. Implementing such strategies is, however, extremely difficult due to our limited understanding of the complex and multifactorial nature of acetic-acid tolerance and sensitivity. This provides a strong incentive to explore the potential of evolutionary engineering for increasing acetic-acid tolerance of *S. cerevisiae,* as this approach does not require *a priori* knowledge on the molecular basis of cellular tolerance.

The xylose-fermenting *S. cerevisiae* strain RWB 218 used in this study was derived previously from the laboratory strain CEN.PK through a combination of metabolic engineering and evolutionary engineering (Kuyper et al. 2005). As observed in other xylose-fermenting *S. cerevisiae* strains, the kinetics of xylose fermentation are strongly affected by the presence of acetic acid at low pH, especially in the absence of glucose (Bellissimi et al. 2009). The goal of the present study was to investigate whether acetic-acid tolerance of an engineered, xylose fermenting *S. cerevisiae* strain can be increased via evolutionary engineering in two different experimental set-ups: (i) sequential anaerobic, pH controlled batch cultivation on xylose at gradually increasing concentrations of acetic acid and (ii) prolonged cultivation in anaerobic, xylose-grown and acetic-acid supplemented continuous cultures without pH control, in which acidification due to the

consumption of ammonium provides a continous selection pressure for cells with improved acetic-acid tolerance.

Materials and Methods

Strains and maintenance. *Saccharomyces cerevisiae* RWB 218 is a genetically and evolutionarily engineered xylose-utilizing strain that expresses the *Piromyces* XylA (xylose isomerase) gene, and in which the enzymes of the nonoxidative pentose-phosphate pathway have been overexpressed (Kuyper et al. 2005). Stock cultures were grown at 30 °C in shake flasks on a synthetic medium supplemented with 20 g L^{-1} glucose. When the stationary phase was reached, sterile glycerol was added to 30% (v/v), and 2-mL aliquots were stored in sterile vials at -80 °C. For storage of the long term selection runs, culture samples were centrifuged, resuspended in synthetic medium supplemented with 30% (v/v) sterile glycerol and stored at -80 °C for further characterization. Material transfer requests for strain RWB 218 should be addressed to Royal Nedalco (info@nedalco.nl, for the attention of J.J. den Ridder).

Cultivation and media. Shake-flask cultivation was performed at 30 °C in a synthetic medium (Verduyn et al. 1992). The pH of the medium was adjusted to 6.0 with 2 M KOH prior to sterilization. Precultures were prepared by inoculating 100 ml medium containing 20 g L^{-1} xylose in a 500 ml shake-flask with a frozen stock culture. After 2 to 3 days incubation at 30 °C in an orbital shaker (200 rpm), this culture was used to inoculate fermentor cultures.

All fermentations were carried out at 30 °C in 2-liter laboratory fermentors (Applikon, Schiedam, The Netherlands) with a working volume of 1 liter. The culture pH was kept at pH 4.0 by automatic addition of 2 M KOH, except for the prolonged selection in continuous culture. Cultures were stirred at 800 rpm and sparged with 0.5 l min^{-1} nitrogen (<10 ppm oxygen). Dissolved oxygen was monitored with an autoclavable oxygen electrode (Applisens, Schiedam, The Netherlands). Synthetic medium (Verduyn et al. 1992) was used containing xylose as the carbon

source, supplemented with 100 µl l^{-1} of silicone antifoam (Sigma, antifoam 204), as well as with anaerobic growth factors ergosterol (0.01 g L^{-1}) and Tween 80 (0.42 g L^{-1}) dissolved in ethanol (Bellissimi et al. 2009), resulting in 11-13 mM ethanol in the medium. To minimize diffusion of oxygen, fermentors were equipped with Norprene tubing (Cole Parmer Instrument Company, Vernon Hills, USA), and the medium vessel was sparged with nitrogen gas during continuous fermentations.

For the sequential batch cultivations, the fraction of CO_2 measured in the effluent gas was used to estimate the specific growth rate of each batch, and the cumulative CO_2 production was used to automatically determine when to remove ~99.5% of the culture broth and refill the fermentor with fresh synthetic medium, which also enabled consistent quantification of batch durations.

For continuous selection (D=0.05 h^{-1}) the pH of the medium was adjusted to 4.25 with KOH, but the pH in the fermentor was not maintained at a constant value. The acetic acid concentration in the supplied medium was periodically increased from an initial concentration of 1 g L^{-1} to a final concentration of 5 g L^{-1}. During the acetic acid gradients, the specific xylose consumption rates were calculated from the xylose mass balance for which the change in the xylose concentration was estimated from the derivative of polynomal spline functions.

To obtain a smoothly increasing acetic-acid concentration gradient in continuous cultures, a gradient mixer consisting of two 20 L medium vessels containing 0 and 19 g L^{-1} of acetic acid, respectively, was connected to steady-state anaerobic xylose-limited cultures at a dilution rate of 0.05 h^{-1} and pH 4. Acetic acid supplemented medium was fed to the medium vessel lacking acetic acid at a flow rate equal to the supply of medium to the culture.

Determination of culture dry weight. Culture samples (10.0 or 20.0 ml) were filtered over preweighed nitrocellulose filters (pore size 0.45 µm; Gelman laboratory, Ann Arbor, USA). After removal of medium, the filters were washed with demineralized water, dried in a microwave oven

(Bosch, Stutgart, Germany) for 20 min at 360 W, and weighed.

Gas analysis. Exhaust gas was cooled in a condensor (2 °C) and dried with a Permapure dryer type MD-110-48P-4 (Permapure, Toms River, USA). O_2 and CO_2 concentrations were determined with an NGA 2000 analyzer (Rosemount Analytical, Orrville, USA).

Metabolite analysis. The supernatant obtained following centrifugation of culture samples was analyzed for xylose, organic acids, glycerol, and ethanol via HPLC analysis on a Waters Alliance 2690 HPLC (Waters, Milford, USA) containing a Biorad HPX 87H column (Biorad, Hercules, USA). The column was eluted at 60 °C with 0.5 g L^{-1} H_2SO_4 at a flow rate of 0.6 ml min^{-1}. Detection was performed using a Waters 2410 refractive-index detector and a Waters 2487 UV detector.

Results

Prolonged repetitive batch cultivation with increasing acetic-acid concentrations. At pH4, anaerobic growth on xylose of *S. cerevisiae* RWB218 is already significantly inhibited at an acetic acid concentration of 1 g L^{-1}, while no growth is observed at acetic-acid concentrations above 2 g L^{-1} (data not shown). To select for cells capable of growth at higher acetic-acid concentrations, RWB 218 was grown anaerobically on xylose in 54 sequential batch reactor (SBR) cultures, covering a total cutivation period of 7 months. Over this period, the concentration of acetic acid was gradually increased from 0 to 6 g L^{-1} by discrete increments of 1 g L^{-1} (Fig. 1). Although the lag phase decreased during the first 6 cycles, which were grown in the absence of acetic acid, the specific growth rate on xylose remained constant at 0.20 h^{-1}. This resulted in a cycle time of 2 d per cycle. Upon addition of 1 g L^{-1} of acetic acid to the culture, the cycle time increased to 4 d and the specific growth rate decreased to 0.14 h^{-1}. During the next 3 cycles at 1 g L^{-1} acetic acid, the specific growth rate increased again to 0.17 h^{-1} and the cycle time was reduced to just over 2 d.

Subsequent increases of the acetic-acid concentration to 2, 3, 4 and 5 g L^{-1}, respectively, resulted in qualitatively similar trends: (i) upon each increase of the acetic-acid concentration, the specific growth rate in the subsequent cycle was reduced and the lag phase extended, resulting in increased cycle times. (ii) during the cycles in between the increases in the acetic-acid concentration, the specific growth rate increased and the cycle time consistently showed a downward trend. Further increasing the acetic-acid concentration to 6 g L^{-1} caused a drastic increase of the lag phase and thereby of the cycle time (Fig. 1). At the end of the 54 batch fermentations, corresponding to over 400 generations based on the culture average, the cycle time had decreased again to 4 days. Compared to the initial cycles grown in the absence of acetic acid, the specific growth rate on xylose was reduced by 3-fold (0.06 h^{-1} at 6 g L^{-1} acetic acid).

Growth-regulating pH feedback in prolonged continuous cultures at increasing acetic-acid

concentrations. For the second selection strategy tested in this study, *S. cerevisiae* RWB 218 was cultivated in an anaerobic xylose-limited continuous culture (D=0.05 hr^{-1}) without pH control. The pH of the ingoing fresh synthetic medium was 4.25. As ammonia, the sole nitrogen source in these cultures, is consumed, protons are released into the medium ($NH_4^+ \rightarrow NH_3 + H^+$), thereby causing a decrease in extracellular pH and a concomitant increase of the undissociated acetic-acid concentration ($pK_a = 4.76$). As soon as the concentration of undissociated acetic acid becomes inhibitory, the specific growth rate will decrease below the dilution rate, resulting in decreased ammonium consumption and an increase of the culture pH due to dilution with fresh medium. This leads to an intrinsic growth-regulating feedback loop that provides a constant selection pressure for cells with a higher tolerance to (undissociated) acetic acid, which can continue to grow and acidify the culture broth when growth of other cells is already inhibited.

At the initial acetic-acid concentration of 1 g L^{-1}, the biomass yield on xylose was just under 0.05 g biomass (g xylose) $^{-1}$, corresponding to a specific xylose-consumption rate of 0.97 g xylose (g biomass)$^{-1}$ h^{-1}, and resulting in a specific ethanol production rate of 0.36 g ethanol (g biomass) h^{-1} (Fig. 2). Over the course of 8 months, representing at least 370 generations, the acetic-acid concentration in the supplied medium was periodically increased, from an initial concentration of 1 g L^{-1} to a final concentration of 5 g L^{-1} (Fig. 2A). These increases initially resulted in increased biomass-specific xylose-consumption rates and reduced biomass yields on xylose, consistent with an increased ATP demand for cellular homeostasis. After 125 d, when an acetic-acid concentration of 4 g L^{-1} was reached, the xylose-consumption rate had increased by 75% from 0.97 to 1.7 g (g biomass·h)$^{-1}$, which is the highest xylose uptake flux hitherto reported for xylose-isomerase based, engineered *S. cerevisiae* (Figure 2). The ethanol production rate had increased by a similar factor from 0.36 to 0.63 g ethanol (g biomass)$^{-1}$ h^{-1} (Fig. 2). A further increase of the acetic-acid concentration to 5 g L^{-1} did not result in a further increase of the specific xylose-consumption rate. Remarkably, this did not result in culture washout but, instead, to a steady-state culture that showed approximately the same xylose-consumption rate as observed in the cultures grown at 4 g L^{-1} of

acetic acid.

Apparent instability of selected phenotypes after storage and cultivation under non-selective conditions. Culture samples taken at the end of the SBR and continuous-culture selection experiments were stored at -80 ºC. Before further characterization, frozen samples were grown on xylose in aerobic shake-flask cultures without added acetic acid. Upon reaching exponential phase, these shake flasks were used to inoculate anaerobic bioreactors in which the conditions were similar to those in the final stages of the selection experiments (20 g L^{-1} xylose, 5 g L^{-1} acetic acid, pH 4; see Materials & Methods). Even after one week, neither growth nor xylose consumption were detected. This suggested that the acetic-acid tolerance acquired as a result of both selection strategies, which enabled growth on xylose at low pH at acetic-acid concentrations where such growth was not observed before, was not stable.

Acetic-acid gradient feeding demonstrates inducible acetic-acid tolerance in selected strains. The apparent loss of the acquired acetic-acid tolerance described above does not necessarily imply that tolerance is completely lost, e.g. as a result of an unstable genetic or epigenetic change. Instead, the acquired tolerance might require induction by acetic acid and thus not be expressed adequately when cells are transferred abruptly from a medium without acetic acid to a medium with a high concentration of acetic acid. To investigate the latter possibility, the parental strain *S. cerevisiae* RWB218 and aliquots from both the SBR and continuous selections runs were tested in anaerobic, xylose-limited and pH-controlled continuous cultures in which the acetic-acid concentration was increased linearly from 0 to 7 g L^{-1} over a period of 8 d (200 h). During the batch phase preceeding the gradient, the specific growth rate of all three cultures was identical at 0.17 h^{-1}. Furthermore, xylose-consumption rates (0.55-0.62 g xylose (g biomass)$^{-1}$ h^{-1}) and biomass yields on xylose (0.08-0.09 g biomass (g xylose)$^{-1}$) were very similar for RWB218 and evolved cultures in xylose-limited chemostat cultures (D = 0.05 h^{-1}) without added acetic acid. Interestingly, the residual xylose

concentration was much lower in chemostat cultures of the continuously evolved culture (0.47 g L^{-1}), compared to the SBR evolved culture (0.82 g L^{-1}) and especially compared to RWB218 (1.30 g L^{-1}). This indicated that both evolution runs resulted in an improved affinity ($q_{s,max}/K_S$ (Button 1993)) for xylose, with the most pronounced improvement occurring in the culture evolved under xylose limitation.

After reaching steady state in the absence of acetic acid, the linear acetic-acid gradient was started (Fig. 3). During the first three days of the acetic acid gradient, the parental strain RWB218, which was not selected for acetic-acid tolerance showed increasing xylose-consumption rates. As expected from Monod kinetics for the limiting nutrient, the residual xylose concentration increased to 5.6 g L^{-1} (Fig. 3A). With less xylose available for fermentation and biomass formation, the ethanol concentration and the culture dry weight decreased. When, after three days, the acetic-acid concentration reached 2.5 g L^{-1}, the specific xylose-consumption rate of RWB218 peaked at 1.0 g xylose (g biomass)$^{-1}$ h^{-1} (Fig. 3D). Subsequently, specific-xylose-consumption rates sharply decreased, reflecting the inability of this strain to deal with high acetic-acid concentrations.

As could be expected from the slightly improved affinity of the SBR-selected culture in xylose-limited chemostat cultures, its residual xylose concentration remained lower during the first three days than in the parental strain RWB218 (Fig. 3B). This resulted in slightly higher ethanol concentrations. However, the specific xylose-consumption rate peaked at almost the same acetic-acid concentration (2.5 g L^{-1}) at a value of 1.1 g_{xylose} $g_{biomass}^{-1}$ h^{-1} and decreased in a pattern that was highly similar to that oberved with the RWB218 strain (Fig. 3D). This indicated that prolonged selection in the SBR cultures did not lead to a stable acetic-acid tolerant phenotype.

The culture selected in the continuous-culture set-up without pH control showed a completely different response to the acetic-acid gradient. During the first three days, it still responded similarly to the other strains, albeit at much lower xylose concentrations due to its improved affinity for xylose. However, where the other cultures demonstrated a sharp peak in the xylose-consumption rate, this culture reached xylose-consumption rate of just above 1.2 g xylose (g

biomass)$^{-1}$ h^{-1} and maintained this flux for the next two days up to acetic-acid concentrations of 5 g L^{-1} (Fig. 3D). Although the xylose-consumption rate remained constant during this period, the residual xylose concentration increased from 1.2 g L^{-1} after three days to 3.0 g L^{-1} after 5 d, indicating an impact of acetic acid on the affinity for xylose. With less xylose available for growth and metabolism, both the biomass concentration and ethanol concentration decreased during this period (Fig. 3C). Although slowly decreasing, the xylose-consumption rate remained above 0.9 g_{xylose} $g_{biomass}^{-1}$ h^{-1}, until an acetic-acid concentration of 6 g L^{-1} was reached. At even higher concentrations the xylose consumption flux rapidly decreased and the culture washed out. These results demonstrate that selection in the continuous cultures without pH control resulted in a stable, acetic-acid-inducible acetic-acid tolerance.

Discussion

Prolonged cultivation of xylose-fermenting *S. cerevisiae* strains at increasing concentrations of acetic acid led to adapted cultures that grew and efficiently fermented xylose at total acetic-acid concentrations of up to 6 g l^{-1} at pH 4. These concentrations were much higher than those that allowed growth of the original xylose-fermenting strain *S. cerevisiae* RWB218. In contrast to previous reports on xylose-fermenting strains that are based on expression of heterologous xylose reductase and xylitol dehydrogenase (Helle et al. 2004), the presence of acetic acid did not result in xylitol formation by the xylose-isomerase based strain *S. cerevisiae* RWB218. This demonstrates that high-rate, high-yield ethanol production from xylose by engineered *S. cerevisiae* in the presence of high acetic-acid concentrations is intrinsically possible. This is an important conclusion for development of yeast-based processes for fermentation of lignocellulosic hydrolysates, in which acetic acid is an important inhibitory compound. Although selection in the sequential batch and continuous cultures led to a similar degree of acetic-acid tolerance, fermentation kinetics during the

selection experiments revealed clear differences.

In the sequential batch cultures, each increase of the acetic-acid concentration caused an initial strong increase of the overall fermentation length. The decrease of the fermentation length in subsequent cultivation cycles was not solely due to an increase of the maximum specific growth rate but also and in particular to changes in the lag phase. Lag phases were unexpected in this cultivation system, since the automated replacement of medium was designed to maintain exponential growth. Their occurrence may be linked to the kinetics of xylose fermentation by *S. cerevisiae* RWB218. Automated medium replacement was initiated when at least 80 % of the initial xylose concentration (20 g l^{-1}) had been consumed, leaving a residual concentration below 6 g l^{-1} (0.04 mM), which is below the K_m for xylose uptake by acetate-unadapted *S. cerevisiae* RWB218 (K_m = 0.1 M, (Kuyper et al. 2005)). The rate of sugar fermentation is a key determinant of acetate tolerance in xylose-fermenting *S. cerevisiae* (Bellissimi et al. 2009). A suboptimal xylose-uptake rate towards the end of each cycle may therefore have led to an increased sensitivity to acetic acid and thus explain the observed lag phases. Consistent with the experimental data (Fig. 1), this effect is expected to be most pronounced when the acetic-acid concentration is increased in a subsequent cycle.

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During the other selection strategy in continuous-culture, a biphasic relation was observed between the acetic-acid concentration in the cultures and the specific rates of xylose fermentation (Fig. 2C). Initially, the specific rate of xylose fermentation increased with increasing acetic-acid concentration, consistent with the key role of ATP in acetic-acid tolerance (Bellissimi et al. 2009). The specific rate of 1.7 g_{xylose} $g_{biomass}^{-1}$ h^{-1} reached at an acetate concentration of 4 g L^{-1} is the highest xylose fermentation rate hitherto reported for engineered *S. cerevisiae*. Surprisingly, although the cultures continued to grow at acetate concentrations above 4 g L^{-1}, the specific rate of xylose concentration did not increase further. This strongly suggests selection for 'passive' mechanisms for acetic-acid tolerance, such as a decreased permeability of the cell envelope or a decreased sensitivity of intracellular targets for acetate inhibition

When samples from both selection experiments were stored at -80 ºC and subsequently pregrown in shake flasks on xylose in the absence of acetic-acid stress, they failed to grow in anaerobic batch cultures (pH 4) supplemented with 5 $g.l^{-1}$ acetic acid. This almost complete loss of the high-level acetic-acid tolerance observed during selection is unlikely to be due to reversion of mutations in view of the limited number of generations (± 10) of non-selective growth. There is a rapidly growing evidence for the occurrence of bi- or multistable phenotypes, even in genetically homogeneous cultures (Veening et al. 2008). Such multistability, which can be a direct consequence of the architecture of regulatory or catalytic networks, could in principle be responsible for the rapid reversion to acetate sensitivity upon a change in growth conditions.

In contrast, when similarly pregrown samples were subjected to a linearly increasing acetic acid-concentration, a drastically increased acetic-acid tolerance was observed for the evolved continuous culture (Fig. 3C). Apparently, selection in the continuous cultures resulted in (hyper)inducible rather than constitutive acetic-acid tolerance. Acetic acid occurs in natural environments of *S. cerevisiae,* and is itself a product of anaerobic yeast metabolism. Indeed, *S. cerevisiae* is known to express inducible tolerance mechanisms, such as those induced by the acetate-induced *HAA1* regulon (Abbott et al. 2008; Fernandes et al. 2005). The inducible acetate tolerance of the continuous-culture selected cells may therefore, for example, have resulted from an increased copy number of such acetate-inducible tolerance genes. Interestingly, evolutionary engineering of *S. cerevisiae* for tolerance to furfural, another inhibitor of yeast metabolism that is formed during lignocellulose hydrolysis, yielded a furfural-tolerant phenotype that was retained during cultivation in the absence of furfural (Heer et al. 2009). Since furfural is formed under non-physiological physicochemical conditions, yeast is unlikely to have evolved specific furfural-inducible resistance mechanisms and evolved resistant phenotypes are more likely to be based on constitutively expressed mutations.

The inducible acetic-acid tolerance obtained in the continuous selection system is impractical from an applied point of view, since incorporation of an acetic-acid adaptation step into

industrial ethanol production processes represents an undesirable complication. However, strains with inducible tolerance, obtained via the continuous selection procedure described in this study, provide an interesting starting point to develop strains with constitutive acetate tolerance, either via classical strain improvement (e.g. mutagenesis and selection) or via reverse engineering of acetic-acid tolerance after analysis of the molecular basis of their inducible tolerance by genome-wide analysis techniques.

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Figure legends

Figure 1. Selection of xylose fermenting *S. cerevisiae* strains for improved acetic-acid tolerance in anaerobic sequential batch cultivation on synthetic medium with 20 g L^{-1} xylose at increasing concentrations of acetic acid (0-6 g L^{-1}) and pH 4. An aerobic xylose grown shake-flask culture of RWB 218 was used as an inoculum for the first batch fermentation. For subsequent fermentations, the cumulative CO_2 production was used to determine the automated removal ~99.5% of the culture broth and refill of the culture with fresh medium. Each point indicates the batch duration (○) and maximum specific growth rate (μ_{max}, ●) of one complete batch fermentation.

Comment [a2]: Note that the symbols are not consistent with the presentation of the results on page 9. Have the open and closed circles in the legend been swapped by mistake?

Figure 2. Selection of xylose fermenting *S. cerevisiae* strains for improved acetic-acid tolerance in anaerobic xylose-limited continuous cultivation without pH control.The physiological parameters represented are: specific xylose consumption rate (●, panel A), total acetic-acid concentration (○, panel A), the biomass yield on xylose (●, panel B) and the specific ethanol production rate (○, panel B).

Figure 3. Impact of acetic-acid gradients in continuous cultivation of xylose-fermenting *S. cerevisiae* strain RWB 218 (panel A) and two cultures selected for improved acetic-acid tolerance in either sequential batch cultivation (panel B) or continuous cultivation without pH control (panel C). Indicated are the culture dry weight (◊), the residual xylose concentration (●) and the observed ethanol concentration (○). The specific xylose-consumption rates (g xylose (g biomass)$^{-1}$ h^{-1}) is indicated in panel D for xylose-fermenting *S. cerevisiae* strain RWB 218 (□) and sequential batch reactor culture (■) or continuous cultivation without pH control (▲). The acetic-acid concentration increased over a period of 8 days from 0 g L^{-1} to 7 g L^{-1}.

1 **Figure 1**

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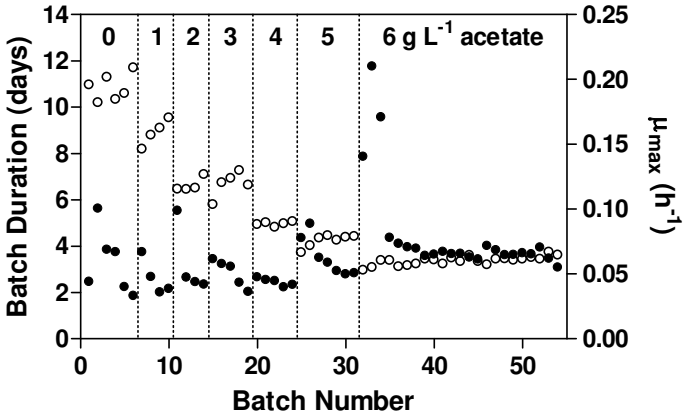


Figure 2

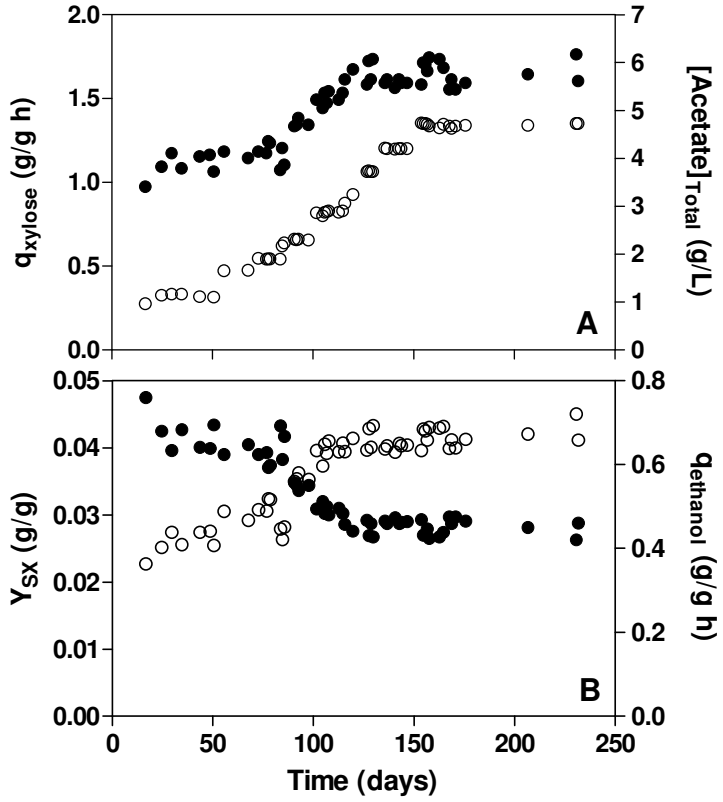
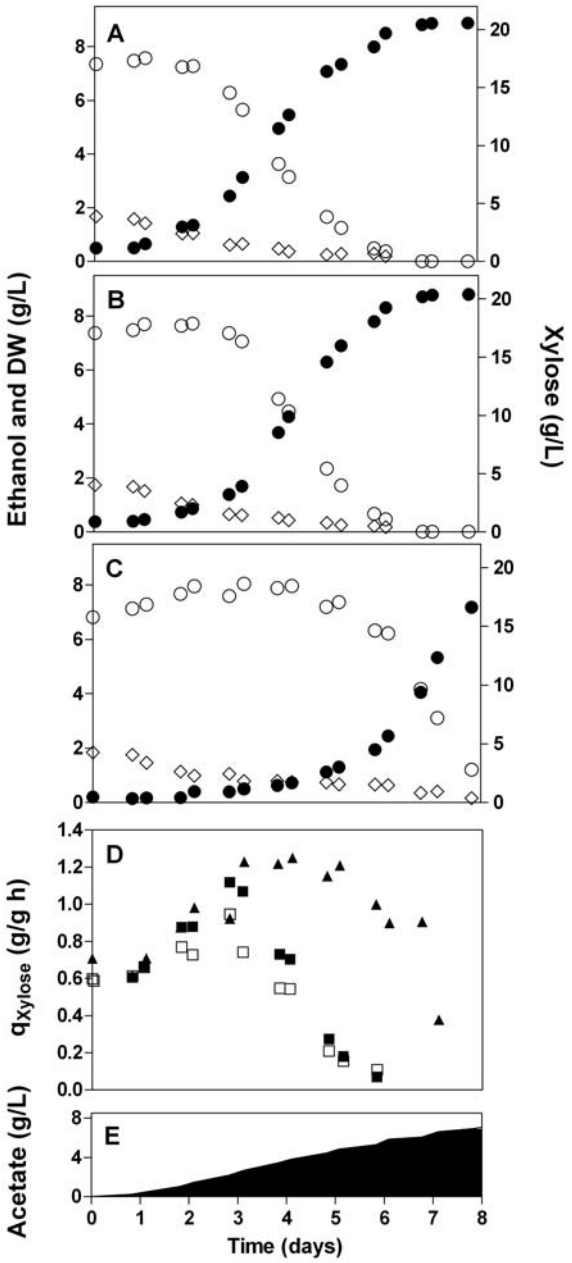

2.0
1.5
1.0
0.5
0.0
q_{xylose} (g/g h)
7
6
5
4
3
2
1
0
$[Acetate]_{Total}$ (g/L)
A
0.05
0.04
0.03
0.02
0.01
0.00
Y_{SX} (g/g)
0.8
0.6
0.4
0.2
0.0
$q_{ethanol}$ (g/g h)
B
0
50
100
150
200
250
Time (days)

Figure 3


A
B
C
D
E
Ethanol and DW (g/L)
Xylose (g/L)
q_{Xylose} (g/g h)
Acetate (g/L)
Time (days)